AWARD NUMBER: W81XWH-13-1-0126

TITLE: Stress and PTSD Mechanisms as Targets for Pharmacotherapy of Alcohol Abuse, Addiction, and Relapse

PRINCIPAL INVESTIGATOR: Dennis Rasmussen, PhD

CONTRACTING ORGANIZATION: Seattle Institute for Biomedical and Clinical Research
Seattle, WA 98108-1532

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	1 2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE

Stress and PTSD Mechanisms as Targets for Pharmacotherapy of Alcohol Abuse, Addiction, and Relapse

5a. CONTRACT NUMBER

Sb. GRANT NUMBER
W81XWH-13-1-0126

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dennis Rasmussen, PhD

E-Mail: drasmuss@u.washinqton.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Seattle Institute for Biomedical and Clinical Research
Seattle, WA 98108-1532

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have demonstrated that alcohol-na"ive rats exhibiting high acoustic startle response (which is associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and alcohol preference in an intermittent alcohol access (IAA) paradigm. The development of increased alcohol intake and increased alcohol preference was highly correlated with acoustic startle amplitude previously determined before the initial access to alcohol. This study, which is key to this entire project, has been published. A new key accomplishment is our recent demonstration that suppression of noradrenergic signaling at the time of traumatic stress decreases acquisition of increased voluntary alcohol drinking long after the stress, which provides a new model for preventive treatment. The schedule for this study was advanced to year 2 instead of the orginally proposed year 3 due to the urgent need for new theerapies to prevent PTSD and alcohol abuse in response to trauma. Preliminary results were recently presented at the 2015 Internatiional Society for PsychoNeuroEndocrinology meeting, and the study will be published after analyses of final data. It has again been necessary to replace a research scientist, causing delays, but all remaining studies and analyses using rat models to address stress and PTSD mechanisms as targets for pharmacotherapy of PTSD and associated alcohol abuse are progressing toward completion as planned.

15. SUBJECT TERMS

PTSD, alcohol, ethanol, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. **INTRODUCTION:** Studies from our research group demonstrating that the well-characterized, safe, well-tolerated and FDA-approved α1-adrenergic receptor antagonist (AR), prazosin, is effective not only in treating combat post-traumatic stress disorder (PTSD) symptoms but in decreasing alcohol drinking in both human and rat studies provide much-needed breakthroughs in the development of effective pharmacotherapies for alcohol use disorders as well as for PTSD. However, much work remains to determine conditions in which this treatment to reduce noradrenergic hyperactivation will be effective, characteristics of individuals who are most likely to respond, and underlying mechanisms providing bases for additional treatments. Our immediate objective is to identify key variables in rat models that will inform and complement human studies, providing a powerful translational approach for most efficiently and rapidly developing and implementing effective new pharmacotherapies for alcohol use disorders and co-morbid PTSD.

2. **KEYWORDS:** alcohol, ethanol, PTSD, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse

3. **OVERALL PROJECT SUMMARY:** There are no significant changes in the project goals or studies planned.
    - **CURRENT OBJECTIVES** There are 3 major goals, or Specific Aims, of this project. These objectives remain unchanged.
        - **1. Determine relationship of hyperexcitability, anxiety and α1-adrenergic receptor-mediated signaling to excessive voluntary alcohol drinking**, *providing information from rat models that will likely reveal especially promising bases for:*

a) Prospectively identifying subsets of individuals who are highly vulnerable to developing alcohol use disorders (AUDs).

STATUS: We have demonstrated that alcohol-naïve rats exhibiting high acoustic startle response (which is associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and alcohol preference in an intermittent alcohol access (IAA) paradigm. These results are consistent with the central hypothesis for all other studies in this research project, i.e., that hyper-responsiveness characteristic of PTSD, alcohol withdrawal/abstinence, and increased noradrenergic activation contributes to – or at least is associated with – development of increased alcohol drinking. This work, corresponding to Task 1, was completed in year 1 and described in detail in the Year 1 progress report. The advance access publication in the journal ALCOHOL AND ALCOHOLISM was also submitted with the Year 1 report and the paper is included in the appendix (Rasmussen and Kincaid, 2015).

b) predicting who is most likely to respond to prazosin with decreased alcohol drinking.

STATUS: This work was not identified as a separate specific Task, but it is a component of several of the proposed experiments. Although our initial work suggests that high acoustic startle and increased anxiety-like behavior is associated with increased suppression of alcohol drinking in response to prazosin, as hypothesized, we are investigating responses over all alcohol access and PTSD-like conditions of this overall investigation, as planned. Consequently, resolution of this issue will not be finalized until all experiments are completed. As described in the original proposal, prazosin is being administered prior to voluntary alcohol drinking in rats that have been previously characterized for acoustic startle and anxiety-like behaviors in the differing experimental models used in these studies; we continue to evaluate whether prazosin treatment disproportionately decreases alcohol drinking in those rats with pre-existing or PTSD-induced high acoustic startle and high anxiety-like behavior.

c) preventing initial acquisition of AUDs in prospectively-identified vulnerable individuals.

STATUS: This work corresponds to Task 2, which was initially predicted to be completed by the end of Year 1 but, as discussed in the Year 1 progress report and in the Statement of Work (SOW) revised at that time, was delayed by implementation of an alternative method of chronic drug administration. The central hypothesis of this experiment is that rats exhibiting high acoustic startle before the initiation of IAA will subsequently exhibit high IAA alcohol intake (as we have demonstrated in Task 1), and that continuous treatment to suppress noradrenergic signaling before and throughout IAA will prevent this acquisition of high alcohol drinking. This experiment thus requires continuous treatment of the rats during introduction of IAA. We had originally proposed to accomplish this with treatments 3 times each day. However, more recent reports suggested that prazosin could be solubilized sufficiently to be administered with implantable osmotic minipumps to maintain prolonged (4 week) constant administration. We confirmed this in an unscheduled determination that the potential use of osmotic minipumps to administer the long-term prazosin treatments was feasible, providing more reliable and consistent drug administration and thus potentially improving results and facilitating extrapolation/translation to humans. Performance of this task (and Task 3, see below) has thus been improved, although final completion of this Task is behind the projected schedule (offset by the advance of Task 6 from year 3 to year 2, as also noted in the Year 1 progress report and associated revised SOW). The thus-improved corresponding experiment has begun, and all initial issues with implementation of the technique have been resolved. The first cohort of 9 rats are now in study, without significant problems, with replicates in larger cohorts to follow. Completion of this study in Year 2 was delayed by unanticipated loss and necessary replacement and training of lab personnel (see discussion of CHANGES, PROBLEMS, DELAYS AND

ACTIONS TO RESOLVE THEM, at the end of this overall project summary). Nonetheless, the most difficult preparation/initiation work for this study is complete, so it could be reasonably estimated that overall progress is 30% complete.

d) predicting who is most vulnerable to progression from voluntary to compulsive alcohol drinking. This work corresponds to Task 3. In the revised SOW provided with the Year 1 progress report, this study was proposed to be conducted in Year 3, which is unchanged. The unscheduled characterization and implementation of the use of osmotic minipumps (discussed above) also provides more reliable and consistent drug administration for this prolonged (28 week) study that requires continuous administration of prazosin for 4 weeks, with an hypothesis that this chronic prazosin will suppress high alcohol drinking even in rats that had exhibited high acoustic startle and anxiety-like behavior before IAA and which had subsequently developed compulsive alcohol drinking (i.e., alcohol drinking that is maintained even after distasteful adulterants are added to the alcohol). The characterizations and methods of the ongoing study *c* (above) are the same as those to be used for this study, which will facilitate efficient completion.

- **2. Evaluate PTSD/alcohol interactions,** *providing information from rat models that will likely reveal especially promising bases for:*

a) determining cause-effect relationships between PTSD and AUDs, i.e., does PTSD increase vulnerability to developing AUDs and do AUDs increase vulnerability to developing PTSD?

STATUS: This work compares production of a PTSD-like behavioral and acoustic startle profile in rats with *vs* without a previous recent history of alcohol liquid diet-induced excessive prolonged alcohol intake and dependence. This work corresponds to Task 4, originally projected to be conducted in year 2, but not yet completed (see discussion of CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM at the end of this overall project summary).

b) predicting who, among individuals with PTSD, is especially vulnerable to developing AUDs.

STATUS: This work addresses whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent acquisition of increased IAA alcohol intake. This work corresponds to Task 5, projected to be conducted in year 3. Nonetheless, it is notable that some of this work has been initiated in year 2 with the study addressing Task 6 (discussed below) which was originally scheduled for year 3 but advanced to start in year 2, as discussed in the Year 1 progress report and in the SOW that was revised and submitted with the Year 1 report. These two studies are being done, in part, in parallel because the methods and time schedules are compatible, with the major difference being that the study addressing Task 6 also incorporates pharmacologic treatment to decrease

noradrenergic signaling at the time of traumatic stress. Consequently, this work is already approximately 75% complete.

c) predicting who, among individuals with PTSD, is likely to respond to prazosin with decreased alcohol drinking.

STATUS: This work, which addresses whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent effectiveness of prazosin in suppressing IAA alcohol intake, was likewise scheduled for year 3. This work was not identified as a separate specific Task with a single proposed completion date, but it is a component of several of the proposed experiments, including Tasks 3, 4, 5, and 6 which are not all projected to be completed until the end of year 3. As described in the grant proposal, prazosin is being administered prior to voluntary alcohol drinking in rats that have been previously characterized for acoustic startle and anxiety-like behaviors in each of the experimental models used in these studies; we continue to evaluate whether prazosin treatment disproportionately decreases alcohol drinking in those rats with pre-existing or PTSD-induced high acoustic startle and high anxiety-like behavior. It is notable that, as with the preceding study, some of this work has been initiated in year 2 with the study addressing Task 6 (discussed below) which was originally scheduled for year 3 but advanced to start in year 2. As with the previous study, these experiments are being done, in part, in parallel because the methods and time schedules are compatible, with the major difference being that the study addressing Task 6 also incorporates pharmacologic treatment to decrease noradrenergic signaling at the time of traumatic stress. This work is approximately 50% complete.

- **3. Determine whether the reduction of α1-AR mediated signaling at the time of traumatic stress will prevent the subsequent development of increased alcohol abuse and PTSD, informing whether prophylactic prazosin treatment is likely to decrease vulnerability to PTSD and alcohol use disorders.**

STATUS: This work includes pharmacologic reduction of noradrenergic signaling at the time of traumatic stress to determine whether this treatment blocks subsequent development of a rat PTSD-like behavioral and acoustic startle profile, as well as increased subsequent IAA alcohol intake. The work corresponds to Task 6, which was originally projected to be conducted and completed in year 3, but – due to urgency of developing effective preventive treatment to block development of PTSD and associated pathologies, such as alcohol use disorders – was advanced to year 2 in the Year 1 progress report and in the associated revised SOW submitted then. Prazosin is administered at the time of traumatic stress to determine whether this treatment blocks subsequent development of a rat PTSD-like behavioral and acoustic startle profile, as well as increased subsequent IAA alcohol intake. However, recent results in some of our unrelated studies (funded by a separate NIH grant) have revealed that the combination of prazosin + the β-adrenergic antagonist,

propranolol, suppresses alcohol drinking and some behavioral responses by alcohol-preferring (P) rats (which, as discussed in our original proposal, exhibit many characteristics similar to PTSD) more effectively than either drug alone, so we also included a comparison group receiving prazosin + propranolol treatment at the time of traumatic stress. Preliminary results with the first 48 male Wistar rats recently were presented at the 2015 Meeting of the International Society for PsychoNeuroEndocrinology (ISPNE), which this year was themed "Stress and the Brain". My presentation was entitled "Reduction of α1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking"; the published abstract is appended to this progress report (Rasmussen et al., 2015). Since this preliminary report was submitted and presented we have completed evaluation of another cohort of 39 rats, for a current total of 87; it is these updated results from 87 rats that are described here. The rats received a single traumatic stress (TS, 10 sec inescapable footshock) or nonstress (NS, 10 sec exposure to shock environment, but without administration of shock) followed by weekly contextual reminders (R) of the TS or NS, as described in detail in the original proposal. Either the α1-adrenergic receptor antagonist prazosin (1 mg/kg), prazosin (1 mg/kg) plus the β-adrenergic receptor antagonist, propranolol (5 mg/kg), or vehicle alone were administered by intraperitoneal (IP) injection at 30-45 minutes before the TS or NS and again at 2 hours after the TS or NS. After 4-5 weekly R followed by 3-4 additional weeks of behavioral testing, the rats were then allowed to voluntarily drink alcohol in an intermittent alcohol access (IAA) model (20% ethanol *vs* water 2-bottle choice access for 24 hours/day on 3 non-consecutive days/week, as discussed in detail in the original grant proposal) for a total of 12 IAA trials (i.e., 3 IAA trials/week for 4 weeks). Alcohol intake was determined in the first 1 hour as well as in all 24 hours of each IAA. The results for the first hour of drinking (thought to reflect motivation to drink alcohol for its acute pharmacologic effects, rather than drinking for caloric content and other factors affecting 24 h drinking) for the 87 rats (i.e., results from the initial 49 rats presented at ISPNE as well as 39 rats completed since then; each group contains equal numbers of individual rats assigned - in a counterbalanced manner based on initial acoustic startle response - to treatment groups within each cohort) are shown in Fig. 1 (next page).

Alcohol intake in the first hour as well as in all 24 hours of each of the IAA trials increased gradually but irregularly in the 12 successive IAA trials ($p<0.001$ by 2-way ANOVA with repeated measures on IAA trial), consistent with the gradual increase in alcohol drinking previously reported for the IAA model. We currently have another cohort of 36 rats under study that have received TS *vs* NS and have begun to receive weekly contextual reminders. When this cohort completes the reminders, post-PTSD model behavioral characterizations, and IAA trials (for a total of 12 weeks post-TS/NS) we will compile and analyze the total data from all

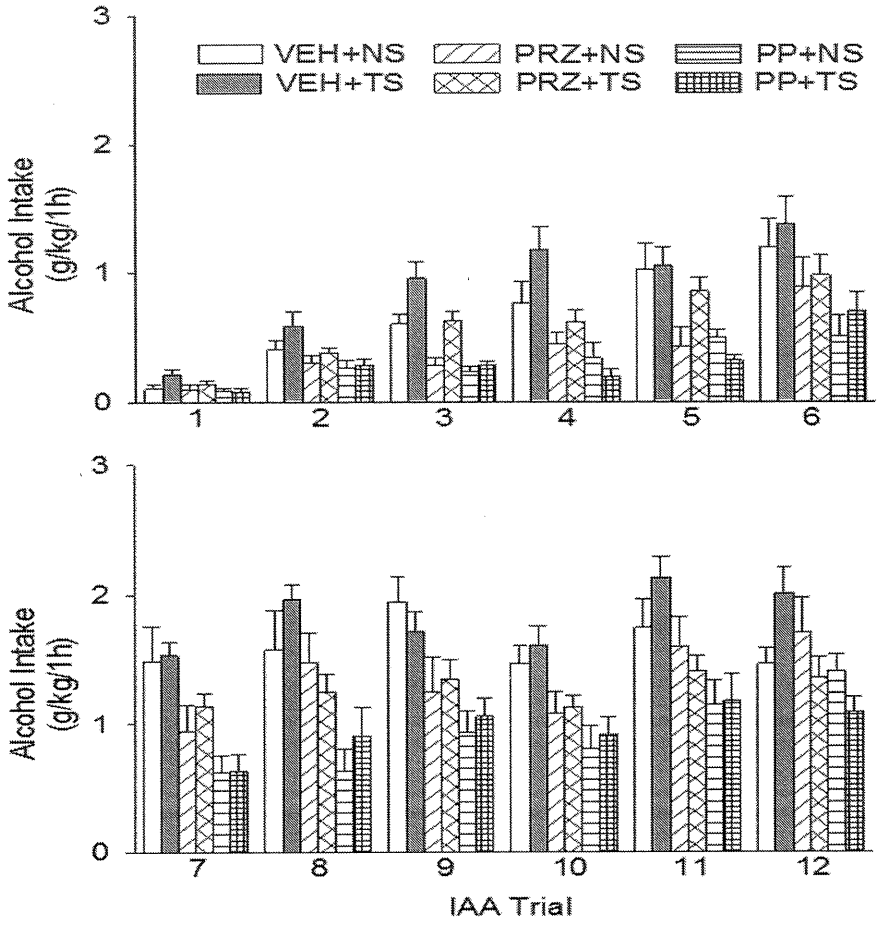

Fig. 1. *Development of increased alcohol intake during the first hour in each of 12 intermittent alcohol access (IAA) sessions following a single 10 second episode of traumatic stress (TS) or no stress (NS) with weekly contextual reminders (R) and subsequent behavioral testing spanning a total of 8 weeks: effect of prazosin (PRZ), prazosin + propranolol (PP) or vehicle (VEH) administered only at the time of TS or NS, 8 weeks prior to initiating IAA. There was one R interposed between IAA trials 6 and 7, so trials 1-6 and 7-12 are plotted separately.*

replicates (i.e., the total 123 rats) for the final characterization of the effects of prazosin or prazosin+propranolol treatment at the time of TS on development of increased alcohol drinking long (8-12 weeks) after the TS. With the complete set of 123 rats we will also analyze the extensive acoustic startle and behavioral test results as discussed in detail in the original proposal, addressing potential correlations and predictive validity of characterizations before and after the rat PTSD model in determining development of a PTSD-like condition and/or increased alcohol intake.

Nonetheless, the preliminary results from the first 87 rats, presented here, already demonstrate significant and intriguing results. Using 2-way ANOVA with repeated measures on IAA trial during the early acquisition of elevated alcohol drinking (IAA trials 1-4), there is significantly higher 1 h alcohol drinking in the rats receiving VEH+TS compared to those receiving only VEH+NS. This trend becomes more variable during the remaining weeks. The commonly elevated alcohol drinking exhibited by the VEH+TS group always tends to be relatively decreased in the PRZ+TS group; this suppression by PRZ is significant ($p<0.05$, 0.01, or 0.001) in 6 of 12 IAA trials and near significant ($0.05<p<0.10$) in 3 more. In contrast, the alcohol drinking exhibited by the traumatic stress group receiving combination treatment with prazosin + propranolol (PP+TS) was consistently suppressed relative to VEH+TS, starting in IAA trial 3 and continuing until IAA trial 12 ($p<0.01$ in each of the 10 IAA trials). These preliminary results from 87 rats are further summarized in Fig. 2, in which the overall daily average (across all 12 IAA trials, i.e., 4 weeks) 1 hour alcohol intakes are analyzed by 2-way ANOVA. With this analysis of overall averages, there

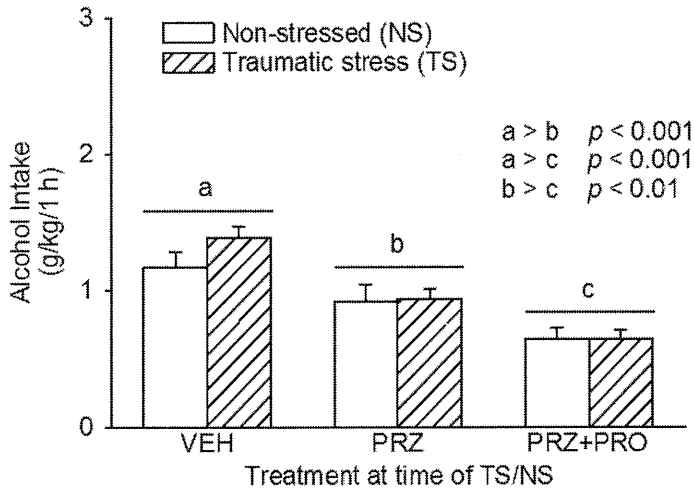

Fig. 2. *Overall daily average (across all 12 IAA trials, i.e., 4 weeks) 1 hour alcohol intakes.*

is a significant main effect of drug treatment ($p<0.001$) but no significant interaction between drug treatment and stress level. PRZ administered at the time of the NS *vs* TS treatment suppressed alcohol drinking during the first hour of each IAA trial more than 8 weeks later ($p>0.001$); this suppression of alcohol drinking was further increased by adding propranolol to the PRZ treatment ($p<0.01$ vs PRZ alone). Results of analyses of 24 hour IAA drinking in these preliminary 87 rats were similar. It should be noted that IAA alcohol drinking in a parallel set of age-matched and co-housed untreated reference rats was lower than the NS rats in this study, suggesting that the experimental conditions experienced

by even the NS rats (IP injections, exposure to bright light in an unfamiliar Gemini box before access to another unfamiliar dark "NS environment" box, exposure to unfamiliar auditory and olfactory cues added at the time of NS, etc) apparently were perceived as somewhat stressful (although not as stressful as the inescapable footshock), as presumably reflected in the suppression of subsequent IAA alcohol drinking by the PRZ and PRZ+PRO treatments at the time of NS treatment in even NS rats.

These preliminary results strongly suggest that a single traumatic stress (TS) followed by weekly contextual reminders can increase voluntary alcohol drinking by male Wistar rats 8-12 weeks after the traumatic stress. Furthermore, treatment with PRZ or PRZ+PRO to reduce noradrenergic signaling at the time of the traumatic stress can prevent this development of increased alcohol drinking, even though the drugs were administered only at the time of the traumatic stress (i.e., 30 minutes before and again 2 hours after the stress) whereas the increased alcohol drinking was expressed 8-12 weeks later. These results thus further suggest that PRZ, PRZ+PRO or other treatments that decrease noradrenergic signaling at the time of traumatic stress could potentially provide prophylaxis for alcohol use disorders that commonly accompany development of PTSD.

The doses and times of PRZ or PRZ+PRO administration before the traumatic stress were the same as the doses and administration times that we have previously demonstrated to acutely and chronically decrease alcohol drinking in rats without producing sedating or motor effects. The route, dose and time of PRZ administration before acute stress were also the same as the dose, route and time of PRZ administration before acute restraint stress that we have previously demonstrated to block stress-induced development of increased anxiety behavior following alcohol withdrawal at least 1 week later. Since increased anxiety can contribute to increased alcohol drinking, the results of that previous study suggest that the effect of decreased noradrenergic signaling at the time of acute stress in the current study may likewise have decreased the later development of increased anxiety, potentially decreasing alcohol drinking.

Analysis of additional cohorts of rats (in progress) will allow more robust resolution of these responses and will allow us to effectively address [TS/NS X drug response] interactions, potential differences in responses to PRZ *vs* PRZ+PRO, characterization of associated PTSD-like acoustic startle and behavioral indices, and further comparisons between VEH+NS rats *vs* age-matched and co-housed untreated reference rats. It is anticipated that the larger overall pooled subject sets will also allow higher resolution characterization of the changes in 24-hour alcohol drinking which is more variable than acute 1-hour drinking, suggested to be due to more accidental spillage associated with rat behavior unrelated to alcohol drinking or to variable confounding by the role that caloric drive can play in alcohol drinking.

This Task is approximately 75% complete.

Anticipated future investigations can also address potential roles for PRZ or PRZ+PRO effects on memory of the TS or on extinction of the response to the TS+R, potential roles of the hypothalamo-pituitary-adrenal axis, effects of administration of PRO alone, effects of PRZ or PRZ+PRO administered at other time points relative to the traumatic stress and/or reminders (e.g., only after the traumatic stress and/or at the time of each reminder) in order to identify additional potential clinically-effective interventions.

- **CHANGES, PROBLEMS, DELAYS AND PLANS TO RESOLVE THEM**

There are no significant changes in objectives and scope. As previously discussed in the Year 1 report, osmotic minipumps are now being used for long-term drug administrations, improving the studies without changing the objectives or scope. The order of the studies was changed in the Year 1 progress report and in the SOW revised at that time, advancing Task 6 to year 2, without changing the objective or scope. As also previously noted in Year 1 report, it took longer than anticipated to recruit, hire (6 months after the award notice), process and train one new staff member (Shelby Johanson) in the first year, introducing delays at the start of the project.

In May, 2015, of Year 2, my long-time Laboratory Manager/Research Scientist, Carrie Kincaid, left my lab for a higher paying position as Research Manager for an extensive clinical research program at this VA medical center. After having her working with me for 10 years, this has of course been disruptive – although she remains available (within the same medical center facility) and willing to assist, if needed, in locating data and records, advising new personnel on methods, etc., and is still co-authoring presentations and papers (such as the recent ISPNE presentation as well as the first paper from this project, in the References section). We have recently recruited, hired, processed and trained her replacement, Jennifer Burns, who has a BS in Physiology with minor in Chemistry and extensive lab research experience, including rodent behavioral testing similar to the testing used in the current studies. Together with the previous need to hire and train Shelby Johanson in year 1, these personnel changes and the time that it has taken to get the new personnel up to speed and operating efficiently has delayed progress. Consequently, it has now become apparent that a no-cost extension of 1 additional year, if possible, would allow more effective completion of all studies as planned, including sufficient time for appropriate thorough analysis, presentation and publication of all results. No increase in overall funding from the grant would be necessary, and there would be no change in the current SOW other than extending the time available to complete all of the studies into a 4th year.

- **SUMMARY DISCUSSION**

The first year was used for personnel recruitment and training, implementation of all necessary methodologies, completion of the first study (which is key to interpreting all subsequent studies), and initiating subsequent studies - each of which is an individually long-term study (ranging from months to greater than a half-year for each of multiple temporally-overlapping cohorts of subjects within each study). In this second year, progress has continued. A second large key study is nearly complete and has already yielded further potentially high impact results which were enthusiastically received when presented at the annual meeting of the International Society of PsychoNeuroEndocrinology, a meeting that this year was focused on effects of stress on the brain. When the final cohort of animals have soon completed the study, analysis of additional PTSD-like behavioral analyses and predictive indices will further extend the impact of this complete study, as planned. In addition, we have improved and initiated several additional studies that are ongoing. The necessary groundwork for successful completion is done and the overall project remains on track, but the start of the project was somewhat slower than predicted and progress has been delayed by another personnel change this year. Consequently we request a 1-year no-cost extension of the project period to allow sufficient time for effective and thorough completion of these important and high impact studies.

4. KEY RESEARCH ACCOMPLISHMENTS

- The key new accomplishment in Year 2 is our demonstration that pharmacologic reduction of noradrenergic signaling at the time of a single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress and long after the brief pharmacotherapy at the time of the trauma. As suggested by my clinical colleague, consultant for these studies, and PTSD investigator, Murray Raskind, MD, in an email response after reading the abstract for the ISPNE presentation: "Wow!! This opens up a whole new prophylaxis world."

5. CONCLUSION:

The key results previously reported for Year 1 were consistent with the hypothesis that is central to all other studies in this research project, i.e. that hyper-responsiveness characteristic of PTSD, alcohol withdrawal/abstinence, and increased noradrenergic activation contributes to development of increased alcohol drinking. These results provided the conceptual basis for a potential approach to prospectively identifying individuals – including individuals with PTSD - at increased risk for future alcohol use disorders, thus allowing development and implementation of potential preventive interventions. The key result from Year 2 now provides evidence for a promising potential preventive intervention.

We have previously demonstrated that a single episode of traumatic stress in our rat PTSD model can produce sustained marked

increases in hyper-responsiveness reflected in acoustic startle response, which is noradrenergic activation dependent; the key result for Year 2, i.e., that reduction of noradrenergic signaling at the time of single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress, demonstrates a potential pharmacologic intervention for preventing at least the subsequently increased alcohol drinking following a traumatic stress. As further behavioral data from the complete study soon become available, we will also evaluate what other aspects of PTSD-like rat behavior also respond to this pharmacologic intervention. The new key finding then also suggests subsequent related questions, such as "would treatment only immediately after a trauma also be effective?", or "would treatment only at the time of each contextual reminder also be effective?", and "what mechanisms are involved?" Addressing these subsequent questions would inform potentially effective pharmacotherapy in cases where traumatic stress had already recently occurred, or perhaps as an adjunct to subsequent PTSD psychotherapy. Together, the key results from Years 1 and 2 also provide the conceptual bases for potential prospective identification of individuals – including individuals with PTSD – at increased risk for future alcohol use disorders, and then potentially applying preventive and – possibly - therapeutic pharmacologic intervention. The remaining studies will likewise further develop a model that will be useful for current and future investigations of neurobiological mechanisms mediating initiation and development of excessive drinking, mechanisms mediating co-morbidity of alcohol use disorders and PTSD, and additional potential treatments of both.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Manuscripts

Lay press: Nothing to report

Peer-Reviewed Scientific Journals:

Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-naïve male rats predicts subsequent voluntary alcohol intake and alcohol preference. Alcohol Alcohol. 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065; PMID: 25305255) [note: this paper was *in press* in the original Year 1 progress report but was later published, so this citation was included when a revised Year 1 progress report was submitted mid-way through Year 2 (the original Year 1 report was unacceptable because I had mistakenly used the new format required for awards issued after 1 October 2013, whereas the award had been issued 30 September 2013).

Invited Articles: Nothing to report

Published abstract:

Rasmussen DD, Johanson SS, Kincaid CL. Reduction of α1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking. Psychoneuroendocrinology 61: 53, 2015 (doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421) Included in appendix.

b. Presentations:

The PI and a PTSD clinical investigator colleague and consultant on this project, Dr. Murray Raskind, together presented a joint seminar at the VA Puget Sound Health Care System (VAPSHCS) Mental Illness Research, Education and Clinical Center (MIRECC) and the VAPSHCS division of Research and Development in affiliation with the Seattle Institute of Biomedical and Clinical Research (SIBCR), entitled "Translation goes both ways; prazosin treatment from humans to rats and back".

The PI presented the work described in the appended published abstract to the 44th Annual Meeting of the International Society for PsychoNeuroEndocrinology – Stress and the Brain, on September 10, 2015.

7. **INVENTIONS, PATENTS AND LICENSES**: Nothing to report

8. **REPORTABLE OUTCOMES:** Our initial key finding in Year 1, that acoustic startle in alcohol-naïve rats is highly predictive of subsequent voluntary IAA alcohol drinking and preference, extends and complements the results of one of our previous studies demonstrating that pre-stress acoustic startle predicts development of rat PTSD-like further increased acoustic startle and plasma corticosterone response following a traumatic stress. Our key finding in Year 2, that reduction of noradrenergic signaling at the time of a single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress, extends and complements our Year 1 key finding by demonstrating a potential new pharmacotherapeutic approach for preventing at least the subsequently increased alcohol drinking following a traumatic stress. Together these results suggest that increased acoustic startle and associated increased anxiety - both of which are increased by noradrenergic activation - reflect underlying mechanisms that increase vulnerability to both PTSD and alcohol abuse. Together with our and others' previous results demonstrating that prazosin can decrease both voluntary alcohol intake and PTSD symptoms, these results strongly suggest that prazosin can be effective for both conditions and that an α1-adrenergic receptor-mediated mechanism is at least one component of the common underlying mechanism, and thus an especially appropriate target for both prophylactic and therapeutic interventions. The remaining studies further investigate these interactions, facilitating most effective translation of

prazosin treatment to clinical utility. All necessary methodology and training has now been developed, trouble-shot and implemented, and these studies are progressing well to successful completion. Our further development of the rat PTSD model, employing a single traumatic stress together with weekly brief contextual reminders of the stress will – together with the further characterization of PTSD-like responses in these studies – also provide a well-characterized experimental model for other labs investigating PTSD and alcohol abuse, alone or together. In addition, a) the findings, results and techniques of these studies are directly applicable to other investigations of the effects of stress or the evaluation of mechanisms contributing to voluntary alcohol and other drug abuse, b) the results of this investigation will facilitate translating prazosin treatment to clinical implementation in the treatment of prazosin and alcohol abuse, alone or together, and c) our results will ultimately improve overall understanding and effective treatment of alcoholism and PTSD, two conditions with profound negative social and economic impact.

9. **OTHER ACHIEVEMENTS:** A great deal of basic and clinical PTSD and related traumatic brain injury (TBI) research is based at the VA Puget Sound Health Care System (VAPSHCS) Mental Illness Research, Education and Clinical Center (MIRECC), providing ample ongoing collaborative opportunities. The PI works closely with a PTSD clinical investigator colleague, Dr. Murray Raskind, as well as alcohol clinical investigators (Drs. Andrew Saxon and Tracy Simpson) and a TBI clinical investigator, Dr. Elaine Peskind, within the VAPSHCS and MIRECC to facilitate translation of basic science findings in the current investigation to clinical testing and future clinical implementation as discussed in Section 8.

10. REFERENCES

Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-naïve male rats predicts subsequent voluntary alcohol intake and alcohol preference. Alcohol Alcohol. 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065; PMID: 25305255)

Rasmussen DD, Johanson SS, Kincaid CL. Reduction of α1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking. Psychoneuroendocrinology 61: 53, 2015 (doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421)

11. **APPENDICES:** One new published abstract and one published paper, listed above in section 6, are appended.

cal pathways involved in sexual desire and function. This paper will present preliminary data suggesting that known neuroendocrine correlates of PTSD are associated with SD. In combat veterans with PTSD ($n = 76$), loss of sexual interest was associated with lower levels of the adrenal androgen DHEA ($\beta = .316$, $t72 = -2.364$, $p = .021$) and plasma cortisol ($\beta = -.378$, $t72 = -3.344$, $p = .001$), and with an attenuated response to the dexamethasone suppression test ($\beta = -.314$, $t67 = -2.565$, $p = .013$). Heightened catecholamines as reflected by the NE/cortisol ratio were associated with problems during sexual relations ($r = .382$, $p = .028$, $df = 31$) and also predicted PTSD intrusive symptoms ($\beta = .216$, $t89 = 2.04$, $p = .044$). In a separate study of treatment seeking veterans with PTSD, urinary NE was associated with difficulty achieving orgasm ($\beta = .340$, $t39 = 2.34$, $p = .024$). In a subsample analysis, testosterone levels did not distinguish SD, suggesting that sexual problems were not the result of organic disorder. Implications and avenues for future research will be discussed.

http://dx.doi.org/10.1016/j.psyneuen.2015.07.533

PO86

HPA axis dysregulation in patients with hypersexual disorder

Andreas Chatzittofis[1,*], Stefan Arver[1], Katarina Öberg[1], Jonas Hallberg[1], Peter Nordström[1], Jussi Jokinen[1,2]

[1] *Karolinska Institutet, Stockholm, Sweden*
[2] *Umeå University, Umeå, Sweden*

Recent focus has been raised on hypersexual disorder that was suggested as a diagnosis for the DSM-5. However, little is known about the neurobiology behind this disorder. A dysregulation of the hypothalamic pituitary adrenal (HPA) axis has been shown in psychiatric disorders but it is not investigated in hypersexual disorder. The aim of this study was to investigate the function of the HPA axis in hypersexual disorder.

The study includes 67 male patients with hypersexual disorder and 39 healthy male volunteers. Basal morning plasma levels of cortisol and ACTH were assessed and low dose (0.5 mg) dexamethasone suppression test was performed with cortisol and ACTH measured post dexamethasone administration. Non-suppression status was defined with DST-cortisol levels ≥138 nmol/l. The Sexual Compulsive scale (SCS), Hypersexual disorder current assessment scale (HD:CAS), Montgomery-Åsberg Depression Scale-self rating (MADRS-S) and Childhood trauma questionnaire (CTQ), were used for assessing hypersexual behavior, depression severity and early life adversity.

Patients with hypersexual disorder were significantly more often DST non-suppressors, had significantly higher DST-ACTH levels and their DST-Cortisol levels showed a trend to be higher compared to healthy volunteers. The patients reported significantly more childhood trauma and depression symptoms compared to healthy volunteers. CTQ scores showed a significant negative correlation with DST-ACTH whereas SCS and HD:CAS scores showed a negative correlation with baseline cortisol in hypersexual patients. The diagnosis of hypersexual disorder was significantly associated with higher plasma DST-ACTH even when adjusted for childhood trauma.

The results suggest a possible HPA axis dysregulation in patients with hypersexual disorder.

http://dx.doi.org/10.1016/j.psyneuen.2015.07.534

PO87

Reduction of α1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking

Dennis Rasmussen[1,2,*], Shelby Johanson[1], Carrie Kincaid[1]

[1] *VISN 20 Mental Illness Research, Education and Clinical Center (MIRECC), VA Puget Sound Health Care System, Seattle, WA, USA*
[2] *Department of Psychiatry, University of Washington, Seattle, WA, USA*

Activation of α1-adrenergic receptor (AR)-mediated mechanisms contributes to post-traumatic stress disorder (PTSD) and to increased acoustic startle response. Pre-stress acoustic startle response (ASR) predicts subsequent increased startle response in a rat PTSD model in which a single traumatic stress (TS, 10 second footshock) is followed by weekly contextual reminders (R) of the TS. ASR also predicts development of increased alcohol drinking in a rat intermittent alcohol access (IAA) model (20% alcohol access for 24 h/day on 3 non-consecutive days/week). We thus hypothesized that reduction of α1-AR signaling at the time of TS would prevent subsequent development of the increased alcohol drinking that commonly accompanies development of PTSD. Male Wistar rats received either TS or no-shock (NS). After 4 weekly R, IAA was provided for 4 weeks. During the final week, average alcohol intake in the first hour of each IAA was increased ($p < 0.05$) in [TS + R] *vs* [NS + R] rats. The α1-AR antagonist, prazosin (PRZ; 1.5 mg/kg), administered IP at the time of the single TS decreased the [TS + R]-induced IAA alcohol drinking ($p < 0.05$, relative to treatment with vehicle), as did PRZ + the β-AR antagonist, propranolol (PRO, 5 mg/kg). These results suggest that PRZ or PRZ + PRO treatment at the time of TS can prevent subsequent development of increased alcohol drinking. We will further address effects of PRZ or PRZ + PRO administered at other time points relative to TS and R, and potential roles of corticosterone. Supported by VA Puget Sound Health Care System, Seattle, Washington and by US Army Medical Research CDMRP W81XWH-13-1-0126.

http://dx.doi.org/10.1016/j.psyneuen.2015.07.535

PO88

Is salivary estriol detectable in very early pregnancy?

Manuela Wingeier[1,*], Pearl La Marca-Ghaemmaghami[1], Roland Zimmermann[2], Ulrike Ehlert[1]

[1] *Department of Clinical Psychology and Psychotherapy, Institute of Psychology, University of Zurich, Zurich, Switzerland*
[2] *Department of Obstetrics, University Hospital Zurich, Department of Obstetrics, University Hospital Zurich, Switzerland*

Background: Estriol is produced in large quantities by the placenta during pregnancy and is important for early pregnancy maintenance. In maternal blood, estriol can be detected from the 8th week of pregnancy and increases sharply after the 10th week under the influence of the HPA axis hormone ACTH. Although estriol can be reliably analyzed in saliva from the 13th week, informa-

Alcohol and Alcoholism Vol. 50, No. 1, pp. 56–61, 2015
Advance Access Publication 10 October 2014

doi: 10.1093/alcalc/agu065

Acoustic Startle in Alcohol-Naïve Male Rats Predicts Subsequent Voluntary Alcohol Intake and Alcohol Preference

Dennis D. Rasmussen* and Carrie L. Kincaid

VISN 20 Mental Illness Research Education and Clinical Center, VA Puget Sound Health Care System and University of Washington, Seattle, WA 98195, USA
*Corresponding author: VA Medical Center, 116-MIRECC, 1660 S Columbian Way, Seattle, WA 98108, USA. Tel.: +1-206-277-3370; Fax: +1-206-768-5456; E-mail: drasmuss@u.washington.edu

(*Received* 19 *June* 2014; *in revised form* 26 *August* 2014; *accepted* 29 *August* 2014)

Abstract — Aims: Acoustic startle response in rats is used to model sensorimotor reactivity. The aim of the study was to determine whether acoustic startle response in alcohol-naïve rats predicts subsequent increased voluntary alcohol drinking or alcohol preference. **Methods:** Startle responses to 90, 95 and 100 decibel (dB) white noise stimuli presented in counterbalanced semi-randomized order were tested in alcohol-naïve young adult male Wistar rats before voluntary alcohol intake was established with an intermittent alcohol access (IAA) model. **Results:** Startle amplitude in response to 95 or 100 dB stimuli was positively correlated with subsequent alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split) pre-IAA startle amplitude in response to 95 or 100 dB stimuli developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA, relative to rats with low pre-IAA startle amplitude. **Conclusion:** Startle response to moderate acoustic stimuli can be a predictive index of vulnerability to developing increased alcohol drinking.

INTRODUCTION

Begleiter and Porjesz (1999) proposed that sensorimotor hyper-reactivity is a key feature of the simplest model of the neuronal milieu underlying a predisposition to alcoholism. This hypothesis is consistent with evidence that sensorimotor hyper-reactivity expressed as enhanced acoustic startle response is characteristic of abstinent alcoholics (Krystal *et al.*, 1997) and is associated with family history of alcoholism (Pfefferbaum *et al.*, 1991; Grillon *et al.*, 1997). Rats selectively bred for alcohol preference and high voluntary alcohol drinking (McKinzie *et al.*, 2000; Chester *et al.*, 2004; Acewicz *et al.*, 2012), and post-dependent rats experiencing either acute alcohol withdrawal or prolonged imposed alcohol abstinence (Rassnick *et al.*, 1992; Rasmussen *et al.*, 2005) also exhibit increased acoustic startle response.

Enhanced startle is associated with increased brain noradrenergic activation (Stevens *et al.*, 1994), and brain noradrenergic activation contributes to increased voluntary alcohol drinking (Walker *et al.*, 2008; Rasmussen *et al.*, 2009; Simpson *et al.*, 2009; Froehlich *et al.*, 2013; O'Neil *et al.*, 2013). Enhanced startle is also correlated with the increased anxiety (Morgan *et al.*, 1993; Davis *et al.*, 1997) that is common to many alcoholics (Cloninger, 1987; Kushner *et al.*, 2000) and that is a major risk factor for alcohol abuse (Koob and Le Moal, 1997). Furthermore, anxiety-related behavior in rats has been demonstrated to predict alcohol drinking under several schedules of alcohol access (Hayton *et al.*, 2012). We thus hypothesized that characterization of startle response may facilitate prospective identification of vulnerability to developing increased voluntary alcohol drinking and also may provide a basis for determining mechanisms mediating development of some alcohol use disorders. Accordingly, we investigated whether prospectively determined acoustic startle response in alcohol-naïve rats was correlated with subsequent increased voluntary alcohol drinking or increased alcohol preference in an intermittent alcohol access (IAA) model. IAA, in which rats have access to 2-bottle choice (water vs 20% alcohol) home cage alcohol drinking for three 24-h sessions/week, separated by at least 24 h (e.g. Monday, Wednesday, Friday), has been reported to induce outbred Wistar rats to escalate alcohol intake over repetitive access sessions to achieve alcohol intake at individually variable high levels accompanied by high alcohol preference and blood alcohol concentrations (BACs) comparable to those achieved by selectively bred alcohol-preferring (P) rats, and has been suggested to effectively model some human alcohol use disorders (Wise, 1973; Simms *et al.*, 2008).

MATERIALS AND METHODS

Animals

Twenty-three alcohol-naïve young adult male Wistar rats (Simonsen Labs, Gilroy, CA, USA) weighing 285 ± 3 g were housed 2/cage in plastic shoebox cages with controlled temperature (21 ± 1°C) and a 12 h/12 h light/dark cycle (lights off at 0900 h). Standard rodent chow (Laboratory Rodent Diet #7001, Harlan Teklad, Madison, WI, USA) and water were available *ad libitum* throughout the study. All experimental procedures were approved by the Veterans Administration Puget Sound Health Care System Institutional Animal Care and Use Committee and conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Acoustic startle testing system

Acoustic startle was tested with an SR-LAB Acoustic Startle System (SDI, San Diego, CA, USA) using a slight modification of methods we previously reported (Rasmussen *et al.*, 2008). Each SR-LAB test chamber includes a ventilated sound-attenuated cabinet containing a clear plastic cylindrical rat enclosure mounted on a piezoelectric accelerometer that detects muscle twitch in response to a brief pulse of white (mixed frequency) noise produced by a tweeter inside the cabinet. The force exerted on the accelerometer is digitized and expressed in millivolt (mV) units. Each startle response signal generated by the accelerometer was recorded as 65 consecutive 1 ms recordings, starting at the onset of each 40-ms startle tone. Results were analyzed as maximum peak

amplitude. Startle stimulus and background white noise levels were calibrated with a Radio Shack Digital Sound Level Meter (33–2055; RadioShack Corp., Fort Worth, TX, USA) placed in the center of the cylindrical rat enclosure. Each of 8 SR-LAB test chambers was calibrated before each use to provide 450 mV response to a consistent test stimulus provided by an SDI Standardization Unit (SDI, San Diego, CA, USA). Each SR-LAB test chamber and rat enclosure was cleaned with 0.5% Liquinox (Alconox, Inc., New York, NY, USA) before each use.

Pre-test acclimation

After acclimation to the animal colony room and the reversed light/dark cycle for 3 weeks, rats were transferred to a dark testing room adjoining the colony room for 90 min at 1.5–3 h after lights-off on each of 5 days prior to testing, with ambient 60 decibel (dB) white noise produced by a White Noise Generator (SDI, San Diego, CA, USA). On the first 4 days, each rat was acclimated to a dark SR-LAB test chamber for 5 min with 60 dB white background noise (produced by the tweeter inside the chamber) before being returned to the colony room, housed with the same cage mate. On the fifth day, each of the rats was likewise transferred to the dark testing room with ambient 60 dB white noise, placed in the dark SR-LAB test chamber for 5 min with 60 dB white background noise, and then exposed to 10 presentations of 40 ms 95 dB white noise pulses presented at 30 s intervals before being returned to the colony room. The goal of this initial exposure to acoustic pulses was to minimize effects of novelty stress in the subsequent acoustic startle testing with similar white noise pulses. Immediately following completion of the acclimation process, each rat was returned to the same colony room but individually housed in a plastic shoebox cage. All procedures during the acclimation and subsequent acoustic startle testing and IAA were conducted under dim red illumination.

Acoustic startle testing

Startle testing was conducted 7–10 days after completion of the pre-test acclimation. On the test day, rats were again transferred to the dark testing room with 60 dB background white noise. After 90 min, each rat was placed in a dark SR-LAB testing chamber with 60 dB background white noise for 5 min before quantitation of startle responses to 10 presentations each of 40 ms 90, 95 or 100 dB white noise pulses at 30 s intervals, with one pulse of each intensity (i.e. 90, 95 or 100 dB) in counterbalanced order within each of 10 sequential sets of three pulses (Table 1), with 60 dB background white noise between pulses. Thus, there were a total of 30 startle tests at 30 s intervals, with 10 tests/each of responses to 90, 95 or 100 dB pulses distributed in counterbalanced order over a 15-min period.

Table 1. Order of acoustic stimuli presentations

Sequential sets of 3 acoustic stimuli	Counterbalanced order of stimuli within sets (dB)		
1	90	95	100
2	95	100	90
3	100	90	95
4	90	95	100
5	95	100	90
6	100	90	95
7	90	95	100
8	95	100	90
9	100	90	95
10	90	95	100

There were 30 s intervals between stimuli within each set as well as between each sequential set.

Alcohol drinking

Three weeks after startle testing, 2-bottle choice access to 20% (v/v) alcohol vs water was provided for 24 h/day, 3 days/week (M, W, F)—i.e. an IAA model (Wise, 1973; Simms *et al.*, 2008). The alcohol solution was prepared by diluting 95% alcohol (ethanol; Decon Labs, King of Prussia, PA, USA) with deionized water to make a 20% (v/v) solution. Alcohol (20%) and water were presented in ball-bearing sipper tubes, with positions of the tubes alternated in sequential alcohol access periods to control for potential side preferences. On days when alcohol was not provided, the rats had access to water only. On days when alcohol and water intakes were characterized for analysis, daily fluid intakes were determined by weighing each tube to the nearest 0.1 g. Alcohol and water tubes were also placed on two empty cages to determine loss due to spillage/leakage and evaporation; average losses in these two cages on each day were subtracted from intakes for that day. Net daily alcohol intake was converted to g alcohol/kg body weight. After 36 alcohol access days (i.e. 12 weeks, when stable alcohol intake was achieved) alcohol intake and alcohol preference (ml of alcohol intake/[ml of alcohol intake + ml of water intake]) were determined over the next 3 alcohol access days to characterize the relationships of each rat's alcohol intake and alcohol preference relative to its pre-IAA acoustic startle responses. One rat did not establish significant daily alcohol drinking (alcohol intake was <1 g/kg/day on all days evaluated) and was excluded from further analyses.

Data analyses

Startle amplitude in response to presentations of 90, 95 or 100 dB acoustic pulse intensities in counterbalanced order within each of 10 sequential sets of 3 pulse presentations were initially evaluated by two-way (set X pulse intensity) repeated measures analysis of variance (ANOVA) with repeated measures on sets (1–10) and pulse intensities (90, 95 or 100 dB). There was a significant effect of intensity, $F(2, 42) = 63.7$, $P < 0.001$, but no significant effect of set and no significant intensity × set interaction. Since startle amplitude in response to each of the acoustic stimulus intensities was independent of presentation time (set) within the 15 min test period, the average of all 10 responses to each stimulus intensity was used in subsequent analyses of the relationships between pre-IAA acoustic startle response vs IAA alcohol intake or alcohol preference. Similarly, IAA alcohol intake or alcohol preference on the 3 alcohol access days in IAA week 13 was analyzed by one-way ANOVA with repeated measures on day; there were no significant effects of day on either alcohol intake or alcohol preference, so 3-day average alcohol intake or 3-day average alcohol preference was likewise used in subsequent analyses of relationships between IAA week 13 alcohol intake or alcohol preference vs pre-IAA acoustic startle amplitude.

Pre-IAA startle amplitude in response to presentations of either 90, 95 or 100 dB stimuli was each compared with subsequent alcohol intake or alcohol preference by Pearson Product Moment Correlation Analysis. The pre-IAA startle response to

90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11$/group) IAA week 13 alcohol intake was further compared by two-way (high vs low alcohol intake X stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The pre-IAA startle amplitude in response to 90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11$/group) IAA week 13 alcohol preference was likewise compared by two-way (high vs low alcohol preference X stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The IAA week 13 alcohol intake or alcohol preference of rats grouped on the basis of high vs low (median split, $n = 11$/group) pre-IAA startle amplitude in response to either 90, 95 or 100 dB stimuli was each analyzed by Student *t*-test (median splits of startle responses to each of the three stimulus intensities did not in each case identify the same animals to be included in the high vs low startle response groups, so two-way ANOVA testing could not be performed); Bonferroni corrections were not applied to individual *t*-tests.

All analyses were conducted using Sigmaplot Version 11 software (Systat Software, Inc., Chicago, IL, USA) with significance accepted at $P < 0.05$. Data are presented as mean ± SEM.

RESULTS

IAA alcohol intake and alcohol preference

Initial (i.e. IAA week 1) average (M, W, F) alcohol intake was 0.95 ± 0.16 g/kg/24 h and alcohol preference was 0.07 ± 0.01. By IAA week 13, alcohol intake had increased to 3.80 ± 0.32 g/kg/24 h ($P < 0.001$) and alcohol preference had increased to 0.40 ± 0.03 ($P < 0.001$).

IAA alcohol intake relative to pre-IAA startle response

IAA week 1 alcohol intake was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.18$), 95 ($P = 0.11$) or 100 ($P = 0.15$) dB stimuli. IAA week 13 alcohol intake relative to pre-IAA startle amplitude elicited in response to presentations of either 90, 95 or 100 dB stimuli is presented in the upper, middle or lower row, respectively, of Fig. 1.

Pre-IAA startle amplitude in response to 90 dB stimuli was modest and inconsistent; startle amplitude was not significantly correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, upper row, left). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol intake revealed no alcohol intake-dependent significant difference in pre-IAA startle in response to 90 dB stimuli (Fig. 1, upper row, center; in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) × stimulus intensity (90, 95,100 dB)] interaction, $F[2, 40] = 5.1$, $P \leq 0.01$, but pre-IAA startle amplitude in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol intake (Fig. 1, upper row, right).

Pre-IAA startle amplitude in response to 95 dB stimuli was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, middle row, left; $P < 0.01$, $r = 0.62$). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol intake in IAA week 13 (Fig. 1, middle row, center; $P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle amplitude in response to 95 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 95 dB stimuli (Fig. 1, middle row, right; $P \leq 0.001$).

Pre-IAA startle amplitude in response to 100 dB stimuli also was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, lower row, left; $P < 0.01$, $r = 0.55$). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, center; $P < 0.001$). Consistent with this results, rats with high (median split) pre-IAA startle response to 100 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, right; $P < 0.01$).

IAA alcohol preference relative to pre-IAA startle response

IAA week 1 alcohol preference was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.19$), 95 ($P = 0.14$) or 100 ($P = 0.20$) dB stimuli.

IAA week 13 alcohol intake and alcohol preference were highly positively correlated, $r = 0.92$, $P < 0.001$. Further analyses of IAA week 13 alcohol preference relationships to pre-IAA startle responses were conducted identically to those in the preceding analysis of alcohol intake relationships to pre-IAA startle responses. Consistent with the high positive correlation between alcohol intake and alcohol preference, the results of analyses of alcohol preference vs pre-IAA startle responses, as detailed below, were essentially identical to the results of the preceding analyses of alcohol intake vs pre-IAA startle responses. Pre-IAA startle amplitude in response to 90 dB stimuli was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.05$, $r = 0.47$). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol preference revealed no alcohol preference-dependent significant difference in pre-IAA startle response to 90 dB stimuli (in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) × stimulus intensity (90, 95, 100 dB)] interaction, $F[2, 40] = 6.41$, $P < 0.01$, but pre-IAA startle in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol preference.

Pre-IAA startle amplitude in response to 95 dB pulses was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.57$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.05$). Consistent with this result, rats with high (median split) pre-IAA startle response to 95 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA low startle response to 95 dB stimuli ($P < 0.01$).

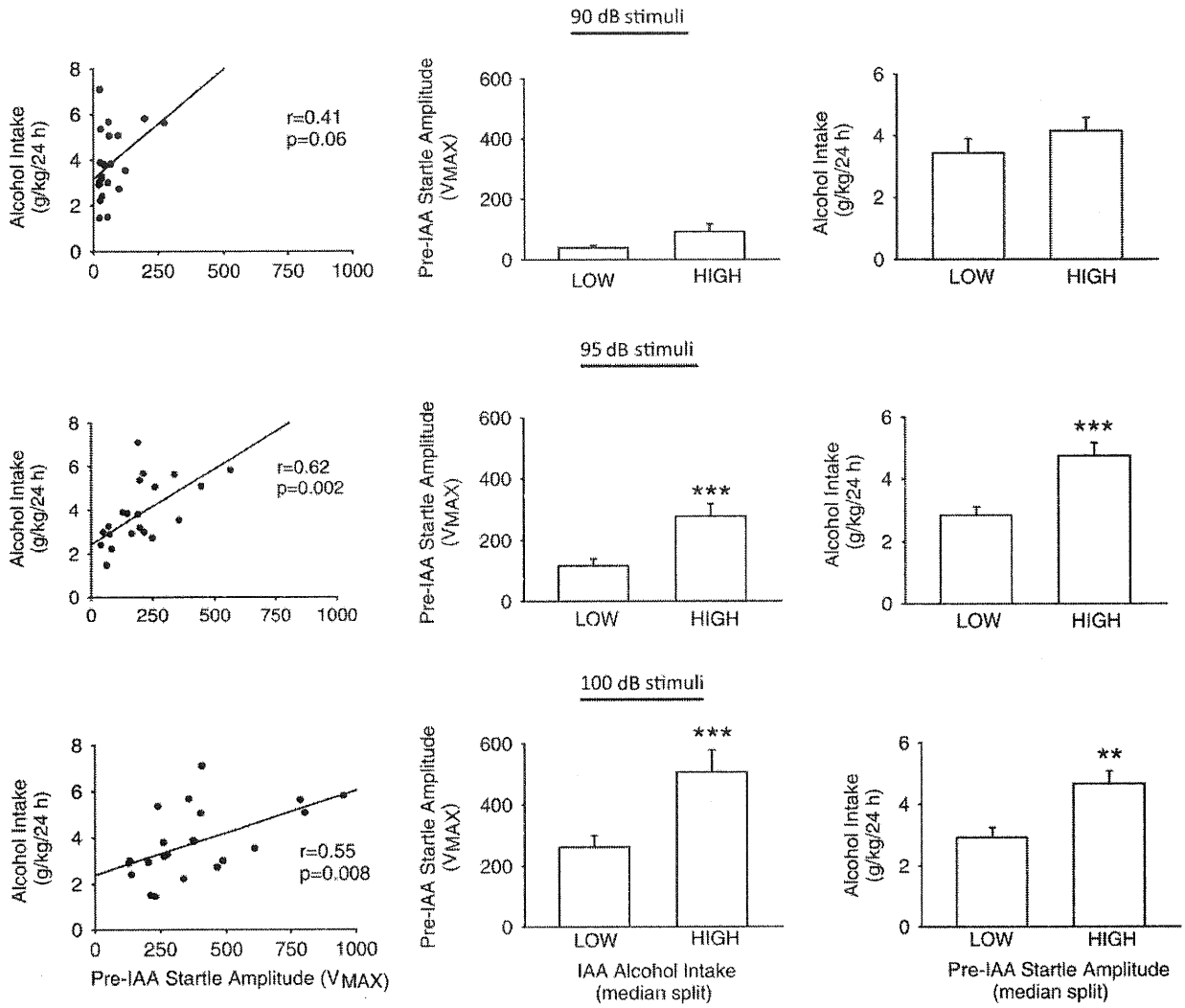

Fig. 1. Pre-IAA acoustic startle response vs alcohol intake following 3 months of IAA. Rows: The upper, middle and lower rows present analyses of pre-IAA responses to 90, 95 or 100 dB acoustic startle stimuli, respectively. Columns: The left panel in each row presents the correlation between pre-IAA acoustic startle amplitude vs IAA week 13 alcohol intake for all 22 rats. The center panel in each row presents the pre-IAA acoustic startle amplitude of rats grouped on the basis of low vs high (median split, $n = 11$ rats/group) alcohol intake in IAA week 13. The right panel in each row presents the IAA week 13 alcohol intake of rats grouped on the basis of low vs high (median split, $n = 11$ rats/group) pre-IAA acoustic startle amplitude. $^{**}P \leq 0.01$ vs Low, $^{***}P \leq 0.001$ vs Low.

Pre-IAA startle response to 100 dB stimuli also was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.53$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to 100 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle response to 100 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli ($P < 0.01$).

IAA alcohol intake relative to pre-IAA startle response to the first presentation of each stimulus intensity

Some previous investigations of alcohol drinking in rats have compared alcohol intake or preference relative to startle amplitude in response to only the first presentation of an acoustic stimulus. In the current study, IAA week 13 alcohol intake or alcohol preference was not significantly correlated with pre-IAA startle in response to the first presentation of either 90 or 100 dB stimuli. However, pre-IAA startle amplitude in response to the first 95 dB stimulus was correlated with IAA week 13 alcohol intake ($r = 0.66$; $P \leq 0.001$) as well as alcohol preference ($r = 0.56$; $P < 0.01$).

DISCUSSION

In alcohol-naïve young adult male Wistar rats, acoustic startle amplitude in response to 40 ms pulses of white noise at intensities of 95 or 100 dB was positively correlated with subsequent voluntary alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split)

alcohol intake or alcohol preference following the 3 months of IAA had previously exhibited greater pre-IAA startle response to 95 as well as 100 dB stimuli, relative to rats with low alcohol intake or low alcohol preference. Conversely, rats with high (median split) pre-IAA startle response to 95 or 100 dB stimuli subsequently developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA.

Stimulus intensities in this investigation were based on the results of preliminary trials with young male Wistar rats in which 90 dB stimuli produced inconsistent small startle responses, 95 or 100 dB stimuli reliably produced relatively consistent sub-maximal startle, and a higher intensity stimulus (120 dB) produced maximal responses. The moderate 90, 95 and 100 dB stimuli were selected in order to avoid ceiling effects that could compromise ability to differentiate responses between animals, as suggested by a report that human startle amplitudes elicited by 90 dB, but not 114 dB, stimuli were positively correlated with number of previous alcohol detoxifications (Krystal *et al.*, 1997). It previously has been reported that male Wistar rats exhibited an inverted U-shaped curvilinear relationship between the startle response to an initial 120 dB acoustic stimulus vs later alcohol intake, and that startle habituation appeared to have predictive value regarding alcohol intake (Sandbak *et al.*, 2000). In the current study, habituation to repeated stimulus exposures was not apparent, and there were significant positive linear correlations between pre-IAA acoustic startle responses to 95 or 100 dB stimuli vs alcohol intake and alcohol preference following IAA. The apparent disparities between the Sandbak *et al.* (2000) study and the current study may be due to the differing stimulus intensities as well as to the incorporation of an initial session with exposure to repetitive moderate (95 dB) stimuli in advance of the testing trial in the current study in order to minimize novelty of the stimulus (consistent with clinical studies, in which the subjects are aware that they will hear acoustic stimuli during the testing trial). In addition, stimuli of three different intensities were presented in semi-random counterbalanced order throughout the 15 min trial in the current study, rather than consistent repetition of a single stimulus. It is also notable that the current study used an IAA model of alcohol drinking in which a relatively high concentration of alcohol (20%, v/v) was available on 3 intermittent days each week, considered to be a model for excessive alcohol drinking (Wise, 1973; Simms *et al.*, 2008).

Alcohol-naïve rats from lines selectively bred to prefer alcohol exhibit increased acoustic startle relative to selectively bred alcohol non-preferring rats (McKinzie *et al.*, 2000; Chester *et al.*, 2004; Acewicz *et al.*, 2012). Sons of alcoholics likewise exhibited increased acoustic startle compared with sons of non-alcoholic parents (Grillon *et al.*, 1997). The current results suggest that mechanisms contributing to acoustic startle response have a functional role in the vulnerability to increased voluntary alcohol drinking, and that acoustic startle characterization can provide an index of sensorimotor hyper-reactivity and associated mechanisms that contribute to this increased alcohol drinking. Although these mechanisms remain to be resolved, it has been demonstrated that brain noradrenergic activation increases acoustic startle response (Stevens *et al.*, 1994) and also produces sensorimotor hyperreactivity and anxiety (Redmoond and Huang, 1979; Sullivan *et al.*, 1999) which are major risk factors for development of alcohol use disorders (Cloninger, 1987; Koob and Le Moal, 1997; Begleiter and Porjesz, 1999; Kushner *et al.*, 2000). Conversely, suppression of noradrenergic signaling not only decreases acoustic startle responses (Gresack and Risbrough, 2011; Olson *et al.*, 2011) but also decreases alcohol drinking in rats and humans (Walker *et al.*, 2008; Rasmussen *et al.*, 2009; Simpson *et al.*, 2009; Froehlich *et al.*, 2013; O'Neil *et al.*, 2013) and blocks the expression of increased alcohol drinking in rats selectively bred for alcohol intake (Froehlich *et al.*, 2013). The consistent association of changes in acoustic startle, anxiety and increased alcohol drinking with changes in noradrenergic signaling suggests that noradrenergic activation may have a key role in mediating the correlation between acoustic startle amplitude and subsequent development of increased voluntary alcohol drinking.

The current results demonstrate that acoustic startle amplitude in response to moderately supra-threshold startle stimulus intensities administered to alcohol-naïve male Wistar rats is an effective predictive index for subsequent increased voluntary alcohol intake and alcohol preference in the IAA model. Acoustic startle response may be an especially useful index of the vulnerability to developing increased alcohol drinking because it is not dependent upon, and potentially confounded by, interactions with other behaviors. Importantly, acoustic startle is also well-characterized for use in humans (Krystal *et al.*, 1997; Grillon and Baas, 2003; Grillon *et al.*, 1998, 2005), providing translational utility.

These results may provide a useful model for investigating neurobiological mechanisms mediating initiation and development of excessive alcohol drinking, as well as provide the conceptual basis for a potential approach to prospectively identifying individuals at increased risk for future alcohol use disorders, thus allowing potential preventive intervention.

Funding — This work was supported in part by resources from the VA Puget Sound Health Care System, Seattle, Washington; US Army Medical Research Acquisition Activity CDMRP (contract number W81XWH-13-1-0126); and National Institutes of Health (grant number AA10567).

Conflict of interest statement. None declared.

REFERENCES

Acewicz A, Mierzejewski P, Jastrzebska A *et al.* (2012) Acoustic startle responses and prepulse inhibition of acoustic startle responses in Warsaw alcohol high-preferring (WHP) and Warsaw alcohol low-preferring (WLP) rats. *Alcohol Alcohol* **47**:386–9.

Begleiter H, Porjesz B. (1999) What is inherited in the predisposition toward alcoholism? A proposed model. *Alcohol Clin Exp Res* **23**:1125–35.

Chester JA, Blose AM, Froehlich JC. (2004) Acoustic startle reactivity during acute alcohol withdrawal in rats that differ in genetic predisposition toward alcohol drinking: Effect of stimulus characteristics. *Alcohol Clin Exp Res* **28**:677–87.

Cloninger CR. (1987) Neurogenetic adaptive mechanisms in alcoholism. *Science* **236**:410–6.

Davis M, Walker DL, Lee Y. (1997) Roles of the amygdala and bed nucleus of the stria terminalis in fear and anxiety measured with the acoustic startle reflex. Possible relevance to PTSD. *Ann N Y Acad Sci* **821**:305–31.

Froehlich JC, Hausauer BJ, Federoff DL *et al.* (2013) Prazosin reduces alcohol drinking throughout prolonged treatment and blocks the initiation of drinking in rats selectively bred for alcohol intake. *Alcohol Clin Exp Res* **37**:1552–60.

Gresack JE, Risbrough VB. (2011) Corticotropin-releasing factor and noradrenergic signaling exert reciprocal control over startle reactivity. *Int J Neuropsychopharmacol* **14**:1179–94.

Grillon C, Baas J. (2003) A review of the modulation of the startle reflex by affective states and its application in psychiatry. *Clin Neurophysiol* **114**:1557–79.

Grillon C, Dierker L, Merikangas KR. (1997) Startle modulation in children at risk for anxiety disorder and/or alcoholism. *J Am Acad Child Adolesc Psychiatry* **36**:925–32.

Grillon C, Dierker L, Merikangas KR. (1998) Fear-potentiated startle in adolescent offspring of parents with anxiety disorders. *Biol Psychiatry* **44**:990–7.

Grillon C, Warner V, Hille J *et al.* (2005) Families at high and low risk for depression: A three-generation startle study. *Biol Psychiatry* **57**:953–60.

Hayton SJ, Mahoney MK, Olmstead MC. (2012) Behavioral traits predicting alcohol drinking in outbred rats: an investigation of anxiety, novelty seeking, and cognitive flexibility. *Alcohol Clin Exp Res* **36**:594–603.

Koob GF, Le Moal M. (1997) Drug abuse: hedonic homeostatic dysregulation. *Science* **278**:52–8.

Krystal JH, Webb E, Grillon C *et al.* (1997) Evidence of acoustic startle hyperreflexia in recently detoxified early onset male alcoholics: Modulation by yohimbine and m-chlorphenylpiperazine (mCPP). *Psychopharmacology* **131**:207–15.

Kushner MG, Abrams K, Borchardt C. (2000) The relationship between anxiety disorders and alcohol use disorders: a review of major perspectives and findings. *Clin Psychol Rev* **20**:149–71.

McKinzie DL, Sajdyk TJ, McBride WJ *et al.* (2000) Acoustic startle and fear-potentiated startle in alcohol-preferring (P) and -nonpreferring (NP) lines of rats. *Pharmacol Biochem Behav* **65**:691–6.

Morgan CAI, Southwick SM, Grillon C *et al.* (1993) Yohimbine-facilitated acoustic startle reflex in humans. *Psychopharmacology* **110**:342–6.

Olson VG, Rockett HR, Reh RK *et al.* (2011) The role of norepinephrine in differential response to stress in an animal model of posttraumatic stress disorder. *Biol Psychiatry* **70**:441–8.

O'Neil ML, Beckwith LE, Kincaid CL *et al.* (2013) The α1-adrenergic receptor antagonist, doxazosin, reduces alcohol drinking in alcohol-preferring (P) rats. *Alcohol Clin Exp Res* **37**:202–12.

Pfefferbaum A, Ford JM, White PM *et al.* (1991) Event-related potentials in alcoholic men: P3 amplitude reflects family history but not alcohol consumption. *Alcohol Clin Exp Res* **15**:839–50.

Rasmussen DD, Burke B, Crites NJ. (2005) Chronic daily ethanol and withdrawal: melatonin treatment reverses persistently increased acoustic startle response during abstinence. *Alcohol Clin Exp Res* **29**(Suppl):16A.

Rasmussen DD, Crites NJ, Burke BL. (2008) Acoustic startle amplitude predicts vulnerability to develop post-traumatic stress hyperresponsivity and associated plasma corticosterone changes in rats. *Psychoneuroendocrinology* **33**:282–91.

Rasmussen DD, Alexander LL, Raskind MA *et al.* (2009) The α_1-adrenergic receptor antagonist, prazosin, reduces alcohol drinking in alcohol-preferring (P) rats. *Alcohol Clin Exp Res* **33**:264–72.

Rassnick S, Koob GF, Geyer MA. (1992) Responding to acoustic startle during chronic ethanol intoxication and withdrawal. *Psychopharmacology* **106**:351–8.

Redmoond DEJ, Huang YH. (1979) Current concepts. II. New evidence for a locus coeruleus-norepinephrine connection with anxiety. *Life Sci* **25**:2149–62.

Sandbak T, Rimol LM, Jellestad FK *et al.* (2000) Relating acoustic startle reactivity and plasticity to alcohol consumption in male Wistar rats. *Physiol Behav* **68**:723–33.

Simms JA, Steensland P, Medina B *et al.* (2008) Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol Clin Exp Res* **32**:1816–23.

Simpson TL, Saxon AJ, Meredith CW *et al.* (2009) A pilot trial of the alpha-1 adrenergic antagonist, prazosin, for alcohol dependence. *Alcohol Clin Exp Res* **33**:255–63.

Sullivan GM, Coplan JD, Kent JM *et al.* (1999) The noradrenergic system in pathological anxiety: a focus on panic with relevance to generalized anxiety and phobias. *Biol Psychiatry* **46**:1205–18.

Stevens DR, McCarley RW, Greene RW. (1994) The mechanism of noradrenergic alpha-1 excitatory modulation of pontine reticular formation neurons. *J Neurosci* **14**:6481–7.

Walker BM, Rasmussen DD, Raskind MA *et al.* (2008) The effects of α1-noradrenergic receptor antagonism on dependence-induced increases in responding for ethanol. *Alcohol* **42**:91–7.

Wise RA. (1973) Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacologia* **29**:203–10.